New Phytologist

Tetranins: new putative spider mite elicitors of host plant defense

Journal:	*New Phytologist*
Manuscript ID	NPH-MS-2018-28235.R2
Manuscript Type:	MS - Regular Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	Iida, Junya; Tokyo University of Science, Department of Biological Science & Technology Desaki, Yoshitake; Tokyo University of Science, Department of Biological Science & Technology Hata, Kumiko; Tokyo University of Science, Department of Biological Science & Technology Uemura, Takuya; Tokyo University of Science, Department of Biological Science & Technology Yasuno, Fumino; Tokyo University of Science, Department of Biological Science & Technology Islam, Monirul; University of Turin, Plant Biology Maffei, Massimo; University of Turin, Plant Biology Ozawa, Rika; Kyoto University, Center for Ecological Research Nakajima, Tadaaki; Tokyo University of Science, Department of Biological Science & Technology Galis, Ivan; Okayama University, Institute of Plant Science and Resources Arimura, Gen-ichiro; Tokyo University of Science, Department of Biological Science & Technology;
Key Words:	defense response, elicitor, tetranin, two-spotted spider mite (Tetranychus urticae), Phaseolus vulgaris

Tetranins: new putative spider mite elicitors of host plant defense

Junya Iida[1]*, Yoshitake Desaki[1]*, Kumiko Hata[1], Takuya Uemura[1], Ayano Yasuno[1], Monirul Islam[2], Massimo E. Maffei[2], Rika Ozawa[3], Tadaaki Nakajima[1], Ivan Galis[4], Gen-ichiro Arimura[1]

[1]Department of Biological Science & Technology, Faculty of Industrial Science & Technology, Tokyo University of Science, Tokyo 125-8585, Japan

[2]Department of Life Sciences and Systems Biology, Plant Physiology-Innovation Centre, University of Turin, Via Quarello15/A, I-10135 Turin, Italy

[3]Center for Ecological Research, Kyoto University, Otsu 520-2113, Japan

[4]Institute of Plant Science and Resources (IPSR), Okayama University, 2-20-1 Chuo, Kurashiki 710-0046, Japan

Corresponding Author:
Gen-ichiro Arimura
Department of Biological Science & Technology, Faculty of Industrial Science & Technology, Tokyo University of Science, 6-3-1 Niijuku, Katsushika-ku, Tokyo 125-8585, Japan
Tel: 81 3 5876 1467
Fax: 81 3 5876 1639

*These authors contributed equally to the article.

Summary

- The two-spotted spider mite (*Tetranychus urticae*) is a plant-sucking arthropod herbivore that feeds on a wide array of cultivated plants. In contrast to the well-characterized classical chewing herbivore salivary elicitors that promote plant defense responses, little is known about sucking herbivores' elicitors. To characterize the sucking herbivore elicitors, we explored putative salivary gland proteins of spider mites by using an *Agrobacterium*-mediated transient expression system or protein infiltration in damaged bean leaves.
- Two candidate elicitors (designated as tetranin1 [Tet1] and tetranin2 [Tet2]) triggered early leaf responses (cytosolic calcium influx and membrane depolarization) and increased the transcript levels of defense genes in the leaves, eventually resulting in reduced survivability of *Tetranychus urticae* on the host leaves as well as induction of indirect plant defenses by attracting predatory mites. Tet1 and/or Tet2 also induced jasmonate, salicylate and abscisic acid biosynthesis.
- Notably, Tet2-induced signaling cascades were also activated via the generation of reactive oxygen species.

- The signaling cascades of these two structurally dissimilar elicitors are mostly overlapping but partially distinct and thus they would coordinate the direct and indirect defense responses in host plants under spider mite attack in both shared and distinct manners.

Key words: defense response, elicitor, *Phaseolus vulgaris*, tetranin (Tet), two-spotted spider mite (*Tetranychus urticae*).

Introduction

Herbivore oral secretions (OS) contain a suite of "elicitors" that induce plant defense responses (Maffei *et al.*, 2012). For instance, a group of hydroxy fatty acid-amino acid conjugates (FACs), including volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] (Alborn *et al.*, 1997), have been intensively characterized as elicitors from several lepidopteran larvae, crickets and fruit fly larvae (Spiteller & Boland, 2003; Yoshinaga *et al.*, 2007; Mori & Yoshinaga, 2011). When volicitin is applied to mechanically damaged sites of leaves of maize seedlings, the seedlings start to emit volatile organic compounds (VOCs) that attract parasitic wasps, natural enemies of maize herbivores (Alborn *et al.*, 1997). FAC-type elicitors such as volicitin induce depolarization of the plant cell plasma membrane potential (Vm), which corresponds to the opening of voltage-dependent Ca^{2+} channels, to initiate cellular defense responses (Maffei *et al.*, 2007). Depolarization of Vm in *Arabidopsis thaliana* leaves has also been shown to be triggered by a porin-like protein from oral secretions of *Spodoptera littoralis* (Guo *et al.*, 2013).

Other distinct types of OS-derived elicitors have been reported, such as disulfoxy fatty acids (caeliferins) from the American bird grasshopper (*Schistocerca americana*) (Alborn

64 *et al.*, 2007), β-glucosidase from the cabbage white butterfly (*Pieris brassicae*) (Mattiacci
65 *et al.*, 1995), the peptide inceptin from the fall armyworm (*Spodoptera frugiperda*)
66 (Schmelz *et al.*, 2006), and a putative β-galactofuranose polysaccharide from the
67 Egyptian cotton leafworm (*Spodoptera littoralis*) (Bricchi *et al.*, 2012). However, all of
68 these elicitors were obtained from chewing herbivores, and very few elicitors have been
69 characterized from sucking types of herbivores. Until now, a mucin-like salivary protein
70 (NlMLP) of the piercing-sucking insect *Nilaparvata lugens* (Huang *et al.*, 2017;
71 Shangguan *et al.*, 2018) is the only elicitor that has been characterized from a sucking
72 herbivore.
73 The two-spotted spider mite (*Tetranychus urticae*, Acari: Tetranychidae) is an
74 agricultural pest of herbaceous and woody plants. In this study, to mine elicitors from
75 spider mites, we focused on their putative salivary proteins/peptides. *In silico* searches of
76 the *T. urticae* genome database (Grbic *et al.*, 2011) and *in planta* analyses using
77 *Phaseolus vulgaris* allowed us to characterize new elicitors, which we named "tetranins,"
78 that play a significant role in eliciting both direct and indirect defense responses of
79 *Phaseolus vulgaris* host plants mediated through an array of intracellular signaling

activations.

Materials and Methods

Plants and mites

Kidney bean plants (*P. vulgaris* cv. Nagauzuramame) and eggplant plants (*Solanum melongena* cv. Chikuyo) were grown in soil for 2 weeks in climate-controlled rooms at 24 ± 1°C with a photoperiod of 16 h (80 μE m^{-2} s^{-1}).

T. urticae Koch (Acari: Tetranychidae) were reared on detached *P. vulgaris* leaf discs (25 cm^2 each) placed on water-saturated cotton in Petri dishes (90 mm diameter, 14 mm depth) at 24 ± 1°C. Small leaf discs (each 1 cm^2), which were inhabited by ~20 mites and eggs, were collected from the original discs and transferred to fresh leaf discs every 2 weeks for incubation. Adult females (10 days old) after oviposition were used for assays.

Phytoseiulus persimilis, obtained from Arysta Lifescience Corporation (Tokyo, Japan), were reared on spider mite-infested *P. vulgaris* plants at 24 ± 1°C. Fertilized females of both mites 5-10 days after their final molt were used for the experiments.

96

97 *Agrobacterium tumefaciens*-mediated transient expression (agroinfiltration) in *P.*

98 *vulgaris* and *S. melongena* leaves.

99 In order to investigate the candidate salivary gland proteins (SGPs) in functional assays,

100 we relied on variants of SGPs with truncation of the signal peptide (Bos *et al.*, 2010;

101 Villarroel *et al.*, 2016). An open reading frame (ORF) of each SGP cDNA fragment

102 without the signal peptide encoding sequence (Table S1) was amplified with KOD -Plus-

103 Neo DNA polymerase (Toyobo, Osaka, Japan). Each cDNA was cloned into TaKaRa T-

104 Vector pMD20 (Takara Bio, Otsu, Japan) and then inserted into binary vector pMDC32

105 (2x CaMV 35S promoter [35SP]::Gateway (GW) region::*nopaline synthase* terminator

106 [NOST]) using the Gateway cloning system (Thermo Fisher Scientific, Waltham,

107 MA). The resulting plasmid, pMDC32-*SGP*, or pMDC32, was transformed into *A.*

108 *tumefaciens* strain EHA105 by electroporation. The bacteria were first cultured overnight

109 in 5 mL of YEP medium with kanamycin and spectinomycin and were then cultured again

110 in 30 mL of YEP medium with kanamycin and spectinomycin for 2-3 h. Cells were

111 harvested by centrifugation and resuspended in 10 mM MES buffer (pH 5.6) and 10 mM

112 $MgCl_2$. The bacterial suspensions were adjusted to an OD_{600} of 0.6 and washed twice with
113 MES buffer; then, acetosyringone was added to a final concentration of 150 μM. The
114 suspensions (approximately 500 μl) were infiltrated into one of the primary leaves of the
115 individual *P. vulgaris* and *S. melongena* plants using a needleless syringe, yielding an
116 infiltrated area of approximately 3 cm^2. A total of 5 independent areas were infiltrated in
117 each primary leaf, and 5 *P. vulgaris* and 6 *S. melongena* plants were assessed, eventually
118 yielding 25 and 30 infiltrated areas, respectively. One day after bacterial infiltration, a
119 piece of leaf disc (1.8 cm^2) was prepared from each single infiltrated area, and the
120 resulting total of 25 *P. vulgaris* and 30 *S. melongena* discs were used for the biological
121 assays described below.
122 Note that there were no differences in the survival of *A. tumefaciens* carrying plasmids
123 for vector control or salivary gland protein in host leaves 2 days after infiltration (Fig.
124 S1).
125
126 Recombinant tetranin protein preparation
127 The ORF of a truncated variant of each tetranin cDNA lacking the signal peptide (Table

S1) or green fluorescent protein (GFP) was subcloned into the pCold™ GST expression vector (Takara Bio). The recombinant vectors (pCold GST-*Tet1* and pCold GST-*Tet2*) were transformed into *Escherichia coli* BL21-CodonPlus(DE3) to prepare the recombinant tetranin1 (Tet1) and tetranin2 (Tet2) proteins. The resulting bacterial strain was grown to A_{600} of 0.8 at 37°C in 2 l of LB medium with ampicillin at 100 μg mL^{-1}. Cultures were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside and kept overnight at 15°C. Cells were pelleted by centrifugation and resuspended in 50 mL of phosphate-buffered saline (PBS) buffer (pH 7.3). Resuspended cells were lysed by sonication. Cell extracts were clarified by centrifugation, and the soluble proteins in the supernatant were purified using Glutathione Sepharose 4B (GE Healthcare Japan, Tokyo) following the manufacturer's protocol. The glutathione-*S*-transferase (GST) tag was removed using HRV 3C Protease (Takara, Otsu, Japan). The buffer was finally replaced with 20 mM HEPES buffer (pH 7.0) via dialysis using cellulose tubing, 8/32 (Eidia Corporation, Tokyo, Japan) three times: first for 3 h, next overnight, and finally for 3 h.

Protein infiltration

144 Tetranin or GFP proteins (3 µM) dissolved in 500 µl of HEPES buffer were infiltrated
145 into one of the primary leaves of *P. vulgaris* plants with a needleless syringe, yielding
146 approximately 3 cm^2 of infiltrated area per leaf. One day after infiltration, a disc (1.8 cm^2)
147 of the infiltrated area was prepared from each individual leaf for use in the spider mite
148 mortality assays (see below). In addition, a fragment of the infiltrated area (approximately
149 100 mg fresh weight) was harvested for RNA extraction from the leaves at the respective
150 time after infiltration.
151
152 Mechanical damage (MD) and protein application
153 MD was performed with stainless steel needles on the primary leaves of potted plants
154 (with 2-mm intervals between the MD spots). Approximately 20 MD spots were applied
155 onto one of the primary leaves of an individual plant for transcript/hormone analyses.
156 Otherwise, approximately 100 MD spots were applied onto the two primary leaves of an
157 individual plant for volatile/olfactometer analyses (50 MD spots/leaf). Tetranin or GFP
158 proteins (0.1-3.0 µM) dissolved in HEPES buffer were applied onto the MD spots
159 (approximately 1 µL per spot) immediately after MD.

160

161 Spider mite mortality assays

162 Ten adult female spider mites were transferred from a master *P. vulgaris* leaf disc (25

163 cm^2 each, see above) on which approximately 800 mites had been incubated for 10 days,

164 onto a *P. vulgaris* leaf disc (1.8 cm^2) that had been infiltrated with Agrobacterium or

165 protein solution (see above) on wet cotton in a plastic Petri dish (90 mm diameter). Each

166 dish contained 10 discs. The mortality of mites on the discs was determined 2 days after

167 agroinfiltration or 3 days after protein infiltration. Individual mites were scored as "dead"

168 when they did not respond to brushing. Data are shown after normalization by the

169 mortality of mites from control treatment performed on the same day, in order to

170 normalize for the variability of control data obtained on different assay days.

171

172 *In situ* hybridization

173 Adult females of *T. urticae* were fixed in 1:1 hexane and PBS with 0.1% (v/v) Tween 20

174 (PBST) containing 4% (w/v) paraformaldehyde at 4°C for 30 min. After washing in 1:1

175 methanol and PBST, the mites were sonicated in a cleansing bath of 5 mg ml^{-1} protease

176 K for 6 min, followed by post-fixation with 4% (w/v) paraformaldehyde in PBST for 30
177 min. After washing with PBST, the mites were prehybridized in hybridization buffer
178 (50% (v/v) formamide, 4x SSC, 1x Denhardt's solution, 250 µg/mL yeast tRNA (Sigma-
179 Aldrich, St. Louis, MO), 50 µg mL^{-1} heparin, 5% (w/v) dextran sulfate and 0.1% Tween
180 20) at 52°C overnight.
181 For synthesis of the RNA fragments encoding the ORFs of tetranins, pMD20 vectors into
182 which the cDNA fragments isolated as described above were inserted were digested with
183 either BamH1 or KpnI, and the probes were then synthesized from the linearized plasmids
184 using SP6 RNA polymerase (Roche Applied Science, Indianapolis, IN) and DIG RNA
185 labeling mix (Roche Applied Science).
186 The prehybridized mites were hybridized in the hybridization buffer (200 µL) with the
187 probe (2 µL) at 52°C for 48 h. The mites were washed in 2x SSC buffer containing 50%
188 formamide and 0.1% (v/v) Tween 20 6 times at 48°C for 25 min and then washed in
189 PBST containing 0.1% (w/v) bovine serum albumin (BSA) 3 times for 15 min. The mites
190 were incubated with anti-DIG-AP, Fab fragments (Roche Applied Science) diluted
191 1:1000 in PBSTB at 4°C overnight. After washing with Tris-buffered saline (TBS, pH

192 7.5) and TBS (pH 9.5), the hybridization signal was visualized with 1 mg mL^{-1} Fast Red
193 (Sigma-Aldrich) and 5% (w/v) aluminum sulfate in TBS (pH 9.5) at 4°C in the dark for
194 30-60 min. Methanol was used to stop the reaction and eliminate the background signal,
195 and the mites were then treated with 70% glycerol in PBST. After washing with PBST,
196 the mites were mounted on glass slides and observed with a fluorescence microscope
197 IX71 (Olympus, Tokyo, Japan).
198
199 Protein preparation and Western blot analysis
200 Total proteins were extracted from whole bodies of *T. urticae* adult females (300 mites
201 for Tet1 and 20 mites for Tet2) in Laemmli sodium dodecyl sulfate (SDS) sample buffer
202 (20 µL). Otherwise, proteins were extracted with Laemmli SDS sample buffer (200 µL)
203 from a *P. vulgaris* leaf disc (3 x 3 cm) which had been cut out from a whole leaf that had
204 been exposed to a wet mesh with or without 100 *T. utricae* for 7 days, and from which all
205 the mites and eggs had then been removed, as well as leaves that were evenly sprayed
206 with 1 mL of aqueous solution (0.5% (v/v) ethanol) of methyl jasmonate (MeJA, Wako
207 Pure Chemical Industrials, Ltd., Osaka, Japan; 0.1 mM) and/or methyl salicylate (MeSA,
208 Wako; 0.1 mM) or aqueous solution (0.5% (v/v) ethanol), serving as control treatment,
209 for 24 h. Five microliters each of these extracted protein solutions were resolved on a
210 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred onto a PVDF

membrane (ATTO, Tokyo, Japan). Anti-Tet1 and anti-Tet2 antibodies obtained by immunizing rabbits with the respective peptide antigens (GNDAMIMPTTEDE and ESQKELVEFLGTGGKKVADE; Cosmo Bio, Tokyo Japan) were used as the primary antibodies. Unlabeled anti-rabbit IgG (#3678, Cell Signaling Technology, Danvers, MA) and HRP-linked anti-mouse IgG (#7076) antibodies were used as the secondary and tertiary antibodies. The membranes were soaked with SuperSignal West Femto Maximum Sensitivity Substrate (Thermos Fisher Scientific, Waltham, MA, USA), and the signals were detected with an ImageQuant LAS-4000 imaging system (GE Healthcare, Buckinghamshire, UK).

RNA extraction, cDNA synthesis and quantitative polymerase chain reaction (qPCR)

Approximately 100 mg of leaf tissues were homogenized in liquid nitrogen, and the total RNA was isolated and purified using Sepasol®-RNA I Super G (Nacalai Tesque, Kyoto, Japan) following the manufacturer's protocol. Single-stranded cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo), and 0.5 µg of the total RNA was incubated, first at 37°C for 5 min for the DNase reaction and then at 37°C for 15 min for the RT reaction. Real-time PCR was performed using a CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA) with THUNDERBIRD SYBR qPCR Mix (Toyobo) and gene-specific primers (Table S2). The following protocol

was used: an initial polymerase activation of 60 s at 95°C, followed by 45 cycles of 15 s at 95°C and then 30 s at 60°C. Then, a melting curve analysis preset by the instrument was performed. Relative transcript abundances were determined after normalization of the raw signals with the housekeeping transcript abundance of an actin gene (KF569608).

Membrane potentials

Vm was determined according to the method described previously (Bricchi *et al.*, 2010).

Determination of intracellular calcium variations

Calcium Orange dye (stock solution in DMSO, Molecular Probes, Leiden, The Netherlands) was diluted in HEPES buffer to a final concentration of 5 µM. This solution was applied onto *P. vulgaris* leaves attached to the plant as previously reported (Maffei *et al.*, 2004). After a *P. vulgaris* leaf was gently fixed on a slide glass, a drop of 5 µM Calcium Orange solution (approximately 45 µL) was applied and then covered with another slide glass. After 1 h incubation with Calcium Orange, the leaf was mounted on a Leica TCS SP2 (Leica Microsystems Srl, Milan, Italy) multiband confocal laser

246 scanning microscope (CLSM) stage without separating the leaf from the plant to assess
247 the basic fluorescence levels as a control. Then, 5 µL of either 3 µM Tet1 or 3 µM Tet2
248 protein dissolved in 20 mM HEPES buffer (pH 7.0) was applied onto the *P. vulgaris* leaf,
249 and the calcium signature was observed after 30 min according to the method described
250 previously (Mithöfer *et al.*, 2009).
251
252 ROS measurements
253 Leaf discs (0.785 cm^2) prepared from fresh leaves were incubated overnight in distilled
254 water in a 96-well titer plate (one disc per well). The distilled water was replaced with a
255 luminol-based assay system in 50 µL of solution containing 0.2 µM luminol (Wako Pure
256 Chemical Industrials, Ltd., Osaka, Japan) and 20 µg L^{-1} horseradish peroxidase (Sigma-
257 Aldrich), and ROS production was determined after addition of 50 µl of 3 µM tetranin or
258 GFP protein solution in 500 µL of 20 mM HEPES buffer (pH 7.0). Luminescence was
259 immediately measured using a 1420 Luminescence Counter ARVO Light (PerkinElmer,
260 Boston, MA), and the signal integration time was 1 s.
261

Phytohormone analysis

P. vulgaris leaves (60-100 mg fresh weight) were harvested and immediately frozen in liquid nitrogen. Using 2 mL screw cap microtubes (Sarstedt, Tokyo, Japan), the samples were homogenized in FastPrep®-24 (MP Biochemicals, Santa Ana, CA) using five 2.3 mm zirconia beads and 1 ml of ethyl acetate solvent spiked with deuterated internal standards (IS) (10 ng d3-JA, 5 ng d3-JA-Ile, 10 ng d6-ABA, and 20 ng d4-SA). The hormone analysis was performed according to the method described previously (Tzin *et al.*, 2017), with slight modifications.

Y-tube olfactometer

A potted *P. vulgaris* plant whose primary leaves were treated with MD + Tet1, Tet2 or GFP protein solution (see above) was used as the single-odor source. Otherwise, undamaged plants and plants damaged with 100 adult spider mite females for 24 h were used. We then assessed the olfactory responses of *P. persimilis* using a Y-tube olfactometer (3.5 cm inner diameter, 13 cm long for each branch tube and 13 cm long for the main tube). The *P. persimilis* adult females were starved overnight by placing 20

mites in a sealed plastic case containing wet cotton with water. The mites were introduced into the Y-shaped wire inside the olfactometer, and the numbers of predators choosing a plant that had been treated with mechanical damage and Tet1, Tet2 or GFP or an undamaged plant serving as a basic control were recorded. Predators that did not choose within 5 min ("no choice" subjects) were excluded from the statistical analysis. The orientations of the odor-source containers relative to the olfactometer arms were changed after every five bioassays. Assays using 20 predators were carried out as a single replicate in a day. Three replicates (60 predators in all) were carried out on different days. The experiments were performed in a climate-controlled room (24 ± 1°C).

Volatile analysis

Similarly to the procedure used in the olfactometer assay, a potted *P. vulgaris* plant whose primary leaves were treated with MD + Tet1, Tet2 or GFP protein solution was used as the single-odor source. Otherwise, undamaged plants and plants damaged by 100 adult spider mite females for 24 h were used. Volatiles from the potted plants were collected in a glass container (2 L) using Tenax 60/80 (Gerstel GmbH & Co. KG, Mülheim an der

294 Ruhr, Germany) in a laboratory room (24 ± 1°C, under light conditions) for 3 h. Clean air
295 passed through the charcoal filter was drawn into the glass bottle, and VOCs from the
296 headspace of the bottle were collected at a flow rate of 100 mL min^{-1}. *n*-Tridecane (0.1
297 μg), infiltrated into a piece of filter paper (1 cm^2), was added as an internal standard to
298 the glass container at the onset of VOC collection. The collected volatile compounds were
299 identified and quantified by gas chromatography-mass spectrometry according to the
300 method described previously (Rim *et al.*, 2017).
301
302 Statistical analyses
303 We performed one-way ANOVAs with Dunnett's contrasts and Tukey's HSD tests using
304 the multcomp R package in R version 3.4.2 and an online program
305 (http://astatsa.com/OneWay_Anova_with_TukeyHSD/), respectively. A replicated G-
306 test was conducted to evaluate the data from the Y-tube olfactometer analyses.
307
308 **Results**
309 Isolation and transient expression of putative mite salivary gland protein (SGP) genes

310 On the basis of the 18,414 predicted genes in the ORCAE database
311 (http://bioinformatics.psb.ugent.be/orcae/), we selected 90 putatively annotated SGP
312 genes by keyword searches. Since SGPs are delivered into the saliva through the classical
313 eukaryotic endoplasmic reticulum (ER)-Golgi pathway (Bos *et al.*, 2010), we targeted the
314 genes that harbor signal peptide sequences at the *N*-terminus using the SignalP 4.0
315 program (Petersen *et al.*, 2011), which resulted in the selection of 23 genes. Furthermore,
316 we removed the 4 genes that contained a transmembrane domain. Of the remaining 19
317 genes, the cDNAs of 13 genes were successfully amplified using mRNA from adult mites
318 (Table S1), and the corresponding RNAs were truncated so that they lacked a signal
319 peptide sequence at the N-terminus, and individually expressed transiently in *Phaseolus*
320 *vulgaris* leaves using the agroinfiltration system. Among the
321 cDNAs encoding these 13 SGPs, two members, SGP7 (tetur05g09318) and SGP8
322 (tetur08g07240), caused significantly higher mortality of adult mite females than control
323 cDNA in agroinfiltrated *P. vulgaris* leaves (Fig. 1a). Both SGP7 and SGP8 also increased
324 the mortality of adult females in leaves of another host (*S. melongena*) (Fig. 1b).

325 Moreover, since *Agrobacterium* species, including *A. tumefaciens*, are plant pathogens,
326 they may boost plant defense responses (Escobar & Dandekar, 2003). Therefore, instead
327 of agroinfiltration, we assessed the potential defense response by direct infiltration of
328 SGP7 and SGP8 proteins into *P. vulgaris* leaves. The assays confirmed an enhanced
329 number of dead mites in SGP7- or SGP8-infiltrated leaves after 3 days compared to
330 uninfiltrated leaves and leaves infiltrated with green fluorescent protein (GFP) that served
331 as a control protein (Fig. 1c).
332 Based on all these results, we defined SGP7 and SGP8 as candidates of elicitor-like
333 proteins and named them tetranin1 (Tet1) and tetranin2 (Tet2), respectively.
334
335 Homology and tissue localization
336 To explore whether tetranins share similarities to proteins with predicted and
337 characterized functions, we performed BLASTX searches using the ORCAE database
338 (http://bioinformatics.psb.ugent.be/orcae/overview/Tetur) (Fig. S2). We found that
339 among the crustacean homologues exhibiting E-values $<10^{-5}$, none of the characterized
340 proteins have sequence similarities to tetranins. Tet2 shows strong similarity to 4 putative

341 *T. urticae* SGPs (tetur08g08060, tetur03g00030, tetur08g07910 [approximately 90%
342 identities] and tetur08g08090 [77% identity]). Although tetur08g07910 was excluded
343 from our primary research targets in the first *in silico* screening performed in 2014, this
344 protein was subsequently newly annotated as an SGP in the updated version of the
345 ORCAE database (note that 118 SGPs were annotated as SGPs in Feb. 2018). In contrast,
346 Tet1 shows weaker similarity to putative *T. urticae* SGPs, with best scores of only 36%
347 and 37% identity with putative *T. urticae* SGPs tetur24g01580 and tetur13g02580
348 [SGP10], respectively. It also appeared that neither tetranin has similarity to reported
349 Tetranychus effectors (less than 40% identity with Tu84, Te84, Tu28 or Te28) (Villarroel
350 *et al.*, 2016).
351 To confirm the tissue-specific expression of tetranins in adult mites, we performed *in situ*
352 hybridization using antisense RNA probes (Fig. 2a). We observed staining of the anterior
353 prosomal glands in at least one of the three paired spider mite salivary glands (Mothes &
354 Seitz, 1981), indicating that Tet1 is a putative salivary gland protein. However, we cannot
355 exclude the possibility that Tet1 signals were also partly observed in the silk glands. For
356 Tet2, in contrast, other tissues were concomitantly stained (Fig. S3), although this may

have been caused by non-specific cross-hybridization of the Tet2 probe with transcripts of multiple Tet2 family genes (discussed below).

We next explored whether tetranins are really delivered to host plants. Western blot analysis using anti-Tet1 antibody showed the presence of two signals of Tet1 proteins synthesized using the *E. coli* system; note that the smaller-sized signal (about 27 kDa) matches the size estimated from its amino acid sequence (Fig. 2b). However, Tet1 was detected only as the larger-sized protein in the mite body, and no Tet1 signals were detected in either damaged or undamaged host plants. On the other hand, the analysis using anti-Tet2 antibody showed the presence of two signals for Tet2 protein synthesized using the *E. coli* system and a single signal for Tet2 protein in the mite body. In addition to a signal with identical size to the upper signal, a larger-sized Tet2 protein signal was detected in mite-damaged host plants but not undamaged host plants (Fig. 2b).

Leaf defense traits induced by application of tetranin at damaged sites

When spider mites suck plant tissues, various molecules, such as elicitors, are thought to be secreted into the physically damaged zones of the plant tissues. Therefore, we next

373 applied various doses of tetranin solutions to mechanically damaged (MD) leaf tissues.
374 To understand in detail how tetranins boost the defense response in bean leaves, here we
375 focused on the transcriptional regulation of two selected marker defense genes
376 (*pathogenesis-related* [*PR*] genes) that have been shown to be responsive to spider mite
377 attack in bean plants (Arimura *et al.*, 2000). The transcript levels of *PR1* were
378 significantly elevated in leaves treated with MD + Tet1 or Tet2 at 3 μM each, compared
379 to those treated with a MD + GFP control (Fig. 3a). Likewise, the transcript levels of *PR3*
380 were increased in leaves treated with MD + Tet1 at 3 μM and those treated with MD +
381 Tet2 at concentrations greater than 0.5 μM.
382 Next we analyzed the levels of accumulation of jasmonate (JA), its derivative (jasmonoyl
383 isoleucine (JA-Ile)), and salicylate (SA), phytohormones with essential roles in plant
384 defense against spider mites (Ozawa *et al.*, 2000; Ament *et al.*, 2004; Alba *et al.*, 2015;
385 Okada *et al.*, 2015). The endogenous levels of these phytohormones were elevated in
386 leaves treated with MD + Tet1 or Tet2 compared to those treated with a MD + GFP
387 control (3 μM each, Fig. 3b). However, the endogenous levels of abscisic acid (ABA),
388 which can cross-talk with JA signaling (Anderson *et al.*, 2004; Adie *et al.*, 2007; Seo &

389 Park, 2010), were only increased when the leaves were treated with MD + Tet2. Both
390 defense gene transcripts and phytohormone levels were increased in leaves infested with
391 spider mites for 24 h (Figs. 3a and 3b).
392
393 Early cellular responses
394 As described above, not only Vm changes and Ca^{2+} channel opening but also reactive
395 oxygen species (ROS) production is known to be a typical early cellular response to
396 herbivory (Maffei *et al.*, 2007). We therefore assessed these responses in *P. vulgaris*
397 leaves treated with tetranins. Overall, Tet2 triggered the depolarization of Vm, cytosolic
398 Ca^{2+} influx, and the generation of ROS, compared to treatment with a GFP or buffer
399 control (Fig. 4). However, Tet1 triggered Vm depolarization and cytosolic Ca^{2+} influx
400 but not the generation of ROS.
401
402 Induced indirect defenses
403 Indirect defenses against herbivorous mites are plant defense strategies that act by
404 inducing *de novo* biosynthesis of volatiles and emitting the blend of volatiles to attract

carnivorous predatory mites (Sabelis *et al.*, 2007). If tetranins serve as elicitors for indirect defenses, the blend of volatiles released from potted *P. vulgaris* plants whose leaves had been exposed to tetranins would affect the olfactory responses of the predatory mite *Phytoseiulus persimilis*. As expected, the predatory mites showed a preference for spider mite-infested plants compared with untreated plants (Fig. 5a), as previously reported (Tahmasebi *et al.*, 2014). Likewise, when primary leaves were treated with MD + Tet1 or Tet2, the predatory mites were attracted to the treated plants over untreated plants. In contrast, the predatory mites were not attracted to plants treated with MD + GFP serving as a control.

We therefore hypothesized that the release of an array of volatiles was induced in the plants exposed to MD + Tet1 or Tet2, whereas none of the eight major volatiles were significantly released compared to those released by the control plants after 24 h ($P > 0.05$, Fig. 5b). It should be noted, however, that levels of limonene released by plants treated with MD + Tet1 and MD + Tet2 were elevated 3.2 and 5.7 times , respectively, compared with those released by MD + GFP-treated plants. Likewise, the levels of (*E*)-β-caryophyllene released from plants were elevated 3.8 and 5.2 times in response to MD

421 + Tet1 and MD + Tet2 treatment, respectively.

422

423

424 **Discussion**

425 Spider mites use their stylet-like mouthparts to suck chlorophyll and liquid contents from
426 leaf cells. Because collection of the regurgitant from the small spider mite mouthparts is
427 technically difficult, the characterization of elicitors from sucking herbivores has been
428 difficult to achieve. Instead, genome-wide approaches have been a powerful and
429 convenient tool to screen genes of interest (e.g., salivary gland genes), and *in silico*
430 analyses using databases succeeded in characterizing effector proteins/peptides from
431 green peach aphids *T. urticae* and *T. evansi* (Bos *et al.*, 2010; Villarroel *et al.*, 2016).
432 Using a similar strategy, here we characterized two novel elicitor proteins from the two-
433 spotted spider mite (*Tetranychus urticae*), tetranins, that were responsible for eliciting
434 defense responses in host plants. Tet1 and Tet2 were deduced to be distinct SGP members,
435 since they shared only 19% amino acid identity (Fig. S2). They have no particular
436 conserved domains except those of signal peptides, indicating that tetranins are novel

437 members of putative SGPs.

438 Although previous proteome studies on spider mites identified about 90 and 177 putative
439 salivary gland proteins from *T. urticae* and *T. truncates* (Jonckheere *et al.*, 2016; Zhu *et*
440 *al.*, 2018), neither Tet1 nor Tet2 was listed among them. However, we observed that Tet1
441 is expressed predominantly in the anterior podocephalic salivary glands, possibly together
442 with the silk glands, of *T. urticae*, implying that Tet1 is secreted to outside of the body.
443 Moreover, Tet2 appeared to be expressed in multiple tissues, including the salivary glands
444 (Fig. 2a), suggesting that Tet2 has a more general function in mite metabolism besides
445 being a salivary elicitor. Otherwise, it is possible that the antisense probes might cross-
446 hybridize to other types of mRNA, as the Tet2 gene shares very high nucleotide similarity
447 with 3 putative SPGs (see above).
448 Tet2 may be structurally modified inside the mite body and/or host tissues (Fig. 2b).
449 Given the fact that the molecular sizes of the bands detected were shifted to 5~8 kDa
450 larger than predicted, the proteins might be modulated with large molecules such as
451 mono-ubiquitin or glycosides. Although it could be speculated that we observed non-
452 specific detection of *T. urticae*-elicited plant proteins, an identical-sized signal was

453 scarcely observed when *P. vulgaris* leaves were treated with MeJA and/or MeSA (Fig.
454 S5), supporting the possibility that this protein is secreted from mites. On the other hand,
455 Tet1 would be not abundantly or less secreted into the host tissues at low concentrations
456 below the detection levels of the nano-LC MS/MS system and western blot analysis. This
457 would be in accord with the fact that Tet1 was sufficiently abundant to be detected by
458 immunoblot analysis using only an estimated 5 adult mites, but Tet2 was detected using
459 an estimated 75 adult mites, even though antibodies against both peptides are similarly
460 able to detect the tetranin proteins synthesized using the *E. coli* system. Nonetheless,
461 irrespective of whether tetranins are secreted or not from mites, the use of tetranins
462 generated using the *E. coli* system would be an ideal platform for spider mite pest
463 management, beyond the significance in the natural ecosystem.
464 Tetranins allow *P. vulgaris* host leaves to elicit defense responses via early cellular
465 processes including Vm depolarization and cytosolic Ca^{2+} influx, which are known to
466 initiate cellular defense responses (Maffei *et al.*, 2007; Zebelo & Maffei, 2015) (Figs. 4a
467 and 4b). These early responses may precede the activation of JA and SA signaling that
468 enables the concomitant activation of plant defense responses of spider mite-infested

plants, as generally seen in several plant species, including legumes and Solanaceae (Ozawa *et al.*, 2000; Kant *et al.*, 2004; Alba *et al.*, 2015). It was shown recently that a mucin-like protein (NlMLP) elicitor identified from the sucking herbivore *Nilaparvata lugens* activates Ca^{2+} signaling, MEK2 MAP kinase and the JA signaling pathway in *Nicotiana benthamiana* leaves (Shangguan *et al.*, 2018). NlMLPs do not share similarities with tetranins or other *T. urticae* proteins.

The leaf levels of ROS and ABA were shown to be elevated only when *P. vulgaris* leaves were treated with MD + Tet2 (Figs. 3b and 4c), and little is known about their roles in defense against herbivorous arthropods, including spider mites. It has been shown in Arabidopsis that an FAC-induced ROS burst is antagonistic to JA biosynthesis (Block *et al.*, 2017). However, the features of that system may not correspond to those of the system involving tetranins, because we did not observe any differences in the endogenous JA levels between Tet1- and Tet2-exposed *P. vulgaris* leaves (Fig. 3b), even though Tet2 triggered ROS generation but Tet1 did not. Moreover, while ABA signaling has been reported to modulate plant disease resistance by inducing SA-linked *PR* transcript inductions (Seo & Park, 2010) and by suppressing JA-ethylene signaling pathways

485 (Anderson *et al.*, 2004) in Arabidopsis, again, this system may not correspond to the
486 system involving tetranins in *Phaseolus* sp. It is known that ROS mediate ABA signaling
487 for plant cellular signaling and homeostasis (Cho *et al.*, 2009). However, as our
488 preliminary tests have shown that pre-treating leaves with an ABA biosynthesis inhibitor
489 failed to suppress the Tet2-induced increase of *PR* transcripts in *P. vulgaris* leaves (Fig.
490 S4), it still remains unknown whether Tet2 induces the increase of *PR* transcripts via ROS
491 generation and subsequent ABA signaling.
492 Leaf damage by spider mite attacks caused only slightly increased levels of JA, JA-Ile,
493 and ABA, as shown in Fig. 3b. According to (Bensoussan *et al.*, 2016), spider mite
494 feeding occurred continuously from several minutes to more than half an hour, during
495 which time a mite consumed a single mesophyll cell. During the consumption, mites
496 might secrete unidentified effectors that enable the suppression of JA, JA-Ile, and ABA
497 biosynthesis. Also, regarding at least JAs, since SA signaling is well known to be
498 antagonistic to JA biosynthesis and signaling (Okada *et al.*, 2015), the elevated leaf SA
499 levels might suppress the biosynthesis of JAs upon spider mite feeding. Moreover,
500 applying tetranins did not result in release of volatiles whose quantitative composition

501 fully mimicked that of volatiles released from *P. vulgaris* plants in response to spider
502 mite attacks. The increased release of (*Z*)-3-hexenyl acetate, methyl salicylate, and the
503 two homoterpenes [(*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyltrideca-
504 1,3,7,11-tetraene] apparently did not occur in response to tetranin exposure, as compared
505 with the response to a spider mite attack (Fig. 5b). For the induction of those volatiles,
506 additional factors, including uncharacterized elicitors, might be involved. For example,
507 several types of elicitors (FAC-type elicitors, porin-like protein, and β-galactofuranose
508 polysaccharide) serve as oral elicitors of the model herbivore *S. littoralis* (Spiteller &
509 Boland, 2003; Bricchi *et al.*, 2012; Guo *et al.*, 2013), indicating that host plants
510 concomitantly perceive multiple elicitor molecules from a single herbivore pest, and
511 consequently produce defense responses including volatile emissions. Modulation of
512 plant defense responses to *Mythimna loreyi* and *Parnara guttata* by simultaneous
513 recognition of different types of elicitors in rice has also been proposed (Shinya *et al.*,
514 2016).

Overall, we still need to clarify the puzzling nature of tetranins. However, findings from the present pilot study already provide new hints for unraveling the molecular mechanisms involved in the early plant defense responses against spider mite attack.

Acknowledgements

We thank Mr. T. Matsui, Ms. H. Omura, and Mr. T. Masuda (Tokyo University of Science) for help with the agroinfiltration assays, and Ms. H. Nakatani (Okayama University) for the phytohormone analyses. Deuterated jasmonate standards were kindly provided by Dr. H. Matsuura (Hokkaido University). This work was financially supported in part by JSPS KAKENHI to GA (16K07407) and MEXT Grants-in-Aid for Scientific Research on Innovative Areas to GA (18H04630 and 18H04786), and by MEXT as part of the Joint Research Program implemented at the Institute of Plant Science and Resources, Okayama University.

References

Adie BA, Pérez-Pérez J, Pérez-Pérez MM, Godoy M, Sánchez-Serrano JJ, Schmelz EA, Solano R. 2007. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *Plant Cell* **19**(5): 1665-1681.

Alba JM, Schimmel BC, Glas JJ, Ataide LM, Pappas ML, Villarroel CA, Schuurink RC, Sabelis MW, Kant MR. 2015. Spider mites suppress tomato defenses downstream of jasmonate and salicylate independently of hormonal crosstalk. *New Phytol.* **205**(2): 828-840.

Alborn HT, Hansen TV, Jones TH, Bennett DC, Tumlinson JH, Schmelz EA, Teal PEA. 2007. Disulfooxy fatty acids from the American bird grasshopper *Schistocerca americana*, elicitors of plant volatiles. *Proc. Natl. Acad. Sci. USA* **104**(32): 12976-12981.

Alborn HT, Turlings TCJ, Jones TH, Stenhagen G, Loughrin JH, Tumlinson JH. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. *Science* **276**: 945-949.

Ament K, Kant MR, Sabelis MW, Haring MA, Schuurink RC. 2004. Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. *Plant Physiol.* **135**(4): 2025-2037.

Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR, Kazan K. 2004. Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *Plant Cell* **16**(12): 3460-3479.

Arimura G, Tashiro K, Kuhara S, Nishioka T, Ozawa R, Takabayashi J. 2000. Gene responses in bean leaves induced by herbivory and by herbivore-induced volatiles. *Biochem. Biophys. Res. Commun.* **277**(2): 305-310.

Bensoussan N, Santamaria ME, Zhurov V, Diaz I, Grbic M, Grbic V. 2016. Plant-herbivore interaction: dissection of the cellular pattern of *Tetranychus urticae* feeding on the host plant. *Front. Plant Sci.* **7**: 1105.

Block A, Christensen SA, Hunter CT, Alborn HT. 2017. Herbivore-derived fatty-acid amides elicit reactive oxygen species burst in plants. *J. Exp. Bot.*

Bos JI, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA. 2010. A functional

genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (green peach aphid). *PLoS Genet.* **6**(11): e1001216.

Bricchi I, Leitner M, Foti M, Mithöfer A, Boland W, Maffei ME. 2010. Robotic mechanical wounding (MecWorm) versus herbivore-induced responses: early signaling and volatile emission in Lima bean (*Phaseolus lunatus* L.). *Planta* **232**(3): 719-729.

Bricchi I, Occhipinti A, Bertea CM, Zebelo SA, Brillada C, Verrillo F, De Castro C, Molinaro A, Faulkner C, Maule AJ, et al. 2012. Separation of early and late responses to herbivory in Arabidopsis by changing plasmodesmal function. *Plant J.* **73**: 14-25.

Cho D, Shin D, Jeon BW, Kwak JM. 2009. ROS-Mediated ABA Signaling. *J. Plant Biol.* **52**(2): 102-113.

Escobar MA, Dandekar AM. 2003. *Agrobacterium tumefaciens* as an agent of disease. *Trends Plant Sci.* **8**(8): 380-386.

Grbic M, Van Leeuwen T, Clark RM, Rombauts S, Rouze P, Grbic V, Osborne EJ, Dermauw W, Ngoc PC, Ortego F, et al. 2011. The genome of *Tetranychus urticae* reveals herbivorous pest adaptations. *Nature* **479**(7374): 487-492.

Guo H, Wielsch N, Hafke JB, Svatos A, Mithofer A, Boland W. 2013. A porin-like protein from oral secretions of *Spodoptera littoralis* larvae induces defense-related early events in plant leaves. *Insect Biochem. Mol. Biol.* **43**(9): 849-858.

Huang HJ, Liu CW, Xu HJ, Bao YY, Zhang CX. 2017. Mucin-like protein, a saliva component involved in brown planthopper virulence and host adaptation. *J. Insect Physiol.* **98**: 223-230.

Jonckheere W, Dermauw W, Zhurov V, Wybouw N, Van den Bulcke J, Villarroel CA, Greenhalgh R, Grbic M, Schuurink RC, Tirry L, et al. 2016. The salivary protein repertoire of the polyphagous spider mite *Tetranychus urticae*: a quest for effectors. *Mol. Cell Proteomics* **15**(12): 3594-3613.

Kant MR, Ament K, Sabelis MW, Haring MA, Schuurink RC. 2004. Differential timing of spider mite-induced direct and indirect defenses in tomato plants. *Plant Physiol.* **135**(1): 483-495.

Maffei M, Bossi S, Spiteller D, Mithöfer A, Boland W. 2004. Effects of feeding *Spodoptera littoralis* on lima bean leaves. I. Membrane potentials, intracellular calcium variations, oral secretions, and regurgitate components. *Plant Physiol.*

134(4): 1752-1762.

Maffei ME, Arimura GI, Mithoefer A. 2012. Natural elicitors, effectors and modulators of plant responses. *Natural Product Reports* **29**(11): 1288-1303.

Maffei ME, Mithöfer A, Boland W. 2007. Before gene expression: early events in plant-insect interaction. *Trends Plant Sci.* **12**(7): 310-316.

Mattiacci L, Dicke M, Posthumus MA. 1995. β-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl. Acad. Sci. USA* **92**(6): 2036-2040.

Mithöfer A, Boland W, Maffei ME 2009. Chemical ecology of plant-insect interactions. In: Parker J ed. *Annual Plant Reviews: Molecular Aspects of Plant Disease Resistance*. Chichester, UK: Wiley-Blackwell, 261-291.

Mori N, Yoshinaga N. 2011. Function and evolutionary diversity of fatty acid amino acid conjugates in insects. *J. Plant Interact.* **6**: 103-107.

Mothes U, Seitz KA. 1981. Fine structure and function of the prosomal glands of the two-spotted spider mite, *Tetranychus urticae* (Acari, Tetranychidae). *Cell Tissue Res.* **221**(2): 339-349.

Okada K, Abe H, Arimura G. 2015. Jasmonates induce both defense responses and communication in monocotyledonous and dicotyledonous plants. *Plant Cell Physiol.* **56**(1): 16-27.

Ozawa R, Arimura G, Takabayashi J, Shimoda T, Nishioka T. 2000. Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. *Plant Cell Physiol.* **41**(4): 391-398.

Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**(10): 785-786.

Rim H, Uefune M, Ozawa R, Yoneya K, Takabayashi J. 2017. Experience of plant infestation by the omnivorous arthropod *Nesidiocoris tenuis* affects its subsequent responses to prey-infested plant volatiles. *Biocontrol* **62**(2): 233-242.

Sabelis MW, Takabayashi J, Janssen A, Kant MR, van Wijk M, Sznajder B, Aratchige NS, Lesna I, Belliure B, Schuurink RC 2007. Ecology meets plant physiology: Herbivore-induced plant responses and their indirect effects on arthropod communities. In: Ohgushi T, Craig TP, Price PW eds. *Ecological Communities: Plant Mediation in Indirect Interaction Webs*. Cambridge: Cambridge University Press, 188-217.

629 **Schmelz EA, Carroll MJ, LeClere S, Phipps SM, Meredith J, Chourey PS, Alborn**
630 **HT, Teal PEA. 2006.** Fragments of ATP synthase mediate plant perception of
631 insect attack. *Proc. Natl. Acad. Sci. USA* **103**(23): 8894-8899.
632 **Seo PJ, Park CM. 2010.** MYB96-mediated abscisic acid signals induce pathogen
633 resistance response by promoting salicylic acid biosynthesis in *Arabidopsis*. *New*
634 *Phytol.* **186**(2): 471-483.
635 **Shangguan X, Zhang J, Liu B, Zhao Y, Wang H, Wang Z, Guo J, Rao W, Jing S,**
636 **Guan W, et al. 2018.** A mucin-like protein of planthopper is required for feeding
637 and induces immunity response in plants. *Plant Physiol.* **176**(1): 552-565.
638 **Shinya T, Hojo Y, Desaki Y, Christeller JT, Okada K, Shibuya N, Galis I. 2016.**
639 Modulation of plant defense responses to herbivores by simultaneous recognition
640 of different herbivore-associated elicitors in rice. *Sci. Rep.* **6**: 32537.
641 **Spiteller D, Boland W. 2003.** N-(17-Acyloxy-acyl)-glutamines: novel surfactants from
642 oral secretions of lepidopteran larvae. *J. Org. Chem.* **68**(23): 8743-8749.
643 **Tahmasebi Z, Mohammadi H, Arimura G, Muroi A, Kant MR. 2014.** Herbivore-
644 induced indirect defense across bean cultivars is independent of their degree of
645 direct resistance. *Exp. Appl. Acarol.* **63**(2): 217-239.
646 **Tzin V, Hojo Y, Strickler SR, Bartsch LJ, Archer CM, Ahern KR, Zhou S,**
647 **Christensen SA, Galis I, Mueller LA, et al. 2017.** Rapid defense responses in
648 maize leaves induced by *Spodoptera exigua* caterpillar feeding. *J. Exp. Bot.*
649 **68**(16): 4709-4723.
650 **Villarroel CA, Jonckheere W, Alba JM, Glas JJ, Dermauw W, Haring MA, Van**
651 **Leeuwen T, Schuurink RC, Kant MR. 2016.** Salivary proteins of spider mites
652 suppress defenses in *Nicotiana benthamiana* and promote mite reproduction.
653 *Plant J.* **86**(2): 119-131.
654 **Yoshinaga N, Aboshi T, Ishikawa C, Fukui M, Shimoda M, Nishida R, Lait CG,**
655 **Tumlinson JH, Mori N. 2007.** Fatty acid amides, previously identified in
656 caterpillars, found in the cricket *Teleogryllus taiwanemma* and fruit fly
657 *Drosophila melanogaster* larvae. *J. Chem. Ecol.* **33**(7): 1376-1381.
658 **Zebelo AS, Maffei ME. 2015.** Role of early signalling events in plant–insect interactions.
659 *J. Exp. Bot* **66**: 435-448.
660 **Zhu YX, Song YL, Huang HJ, Zhao DS, Xia X, Yang K, Lu YJ, Hong XY. 2018.**
661 Comparative analyses of salivary proteins from the facultative symbiont-infected

662 and uninfected *Tetranychus truncatus*. *Syst. Appl. Acarol.* **23**(6): 1027-1042.
663

664 **Supporting Information**

665 **Table S1** Putative *Tetranychus urticae* salivary gland proteins focused on in this study.

666 **Table S2** Primers used for qPCR analysis.

667 **Fig. S1** Titer of Agrobacterium cells in *Phaseolus vulgaris* host leaves.

668 **Fig. S2** Phylogenetic trees of deduced amino acids of tetranins, other arthropod genes of

669 unknown function, and mite effector genes.

670 **Fig. S3** *In situ* hybridization using the sense probe of tetranin genes in spider mite bodies.

671 **Fig. S4** Effect of an ABA biosynthesis inhibitor on transcript levels induced in leaves by

672 mechanical damage plus tetranins.

673 **Fig. S5** Immunoblot analysis for Tet2 in *Phaseolus vulgaris* leaves treated with methyl

674 jasmonate and/or methyl salicylate or aqueous solution for 24 h.

675 **Figure Legends**

676 **Fig. 1** Screening of spider mite elicitor-like secretory proteins. Adult spider mites were
677 incubated on discs prepared from *Phaseolus vulgaris* (a) and *Solanum melongena* (b)
678 leaves in which the indicated truncated salivary gland protein (SGP) or vector control
679 (VC) was transiently expressed using the agroinfiltration system. The leaf discs infiltrated
680 with MES buffer alone served as a control (Ct). The mortality rate of mites on the disc
681 was determined after 2 days. The means of the mortality numbers relative to those of
682 mites on the control discs with standard errors are shown (n = 25 for *P. vulgaris* and n =
683 30 for *S. melongena*). (c) Similarly, adult spider mite females were incubated for 2 days
684 or 3 days on discs prepared from *P. vulgaris* leaves that had been infiltrated with GFP,
685 SGP7 or SGP8 protein for 1 day. The uninfiltrated leaf discs served as a control (Ct). The
686 means of the mortality numbers relative to those on the control discs with standard errors
687 are shown. Data represent the means and standard errors (n = 9-10). Data marked with
688 asterisks are significantly different from those of the Ct based on an ANOVA with
689 Dunnett's contrasts (***, $P < 0.001$; **, $0.001 \leq P < 0.01$).

690

691

692 **Fig. 2 Localization and secretion of Tetranins.** (a) *In situ* hybridization using the
693 antisense probe of tetranin (Tet1 or Tet2) in the bodies of adult female spider mites. The
694 merged bright field and fluorescence images are shown. The fluorescence signals
695 observed in the anterior salivary glands are indicated by arrows. Note that the sense RNA
696 probes serving as controls showed staining of the mites' whole bodies (Fig. S3). (b)
697 Immunoblot analysis for Tet1 (top panel) and Tet2 (bottom panel) in the bodies of adult
698 female spider mites, on undamaged and damaged *Phaseolus vulgaris* leaf discs. The
699 putative tetranin signals are indicated by arrows.

700

701 **Fig. 3** Transcript and phytohormone levels in leaves exposed to mechanical damage (MD)
702 and tetranins. (a) Transcript levels of defense genes (*PR1* and *PR3*) in *Phaseolus vulgaris*
703 leaves 24 h after application of MD + GFP (3 μM) or tetranin (Tet1 or Tet2) protein at
704 0.1, 0.5, 1.0 or 3.0 μM and 24 h after exposure to 100 spider mites. Untreated leaves at
705 the same time points served as a control (Ct). Note that we set 24 h as the time point for
706 analysis according to the results of our preliminary time-course analysis of *PR1* and *PR3*

transcript levels in leaves infiltrated with Tet2 (Fig. S4). Data represent the means and standard errors (n = 5-6). Data marked with an asterisk are significantly different from those of MD + GFP based on an ANOVA with Dunnett's contrasts (***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$). Otherwise, the means followed by a P-value are marginally different. (b) Endogenous levels of jasmonic acid (JA), jasmonoyl isoleucine (JA-Ile), salicylic acid (SA) and abscisic acid (ABA) in leaves 1 and 3 h after treatment with MD + GFP, or MD + tetranin proteins (3 µM each), and 24 h after exposure to 100 spider mites. Data represent the means and standard errors (n = 6). The means indicated by different small letters are significantly different based on an ANOVA with post hoc Tukey's HSD ($P < 0.05$); ns, not significant.

Fig. 4 Early cellular responses to tetranins (Tet1 and Tet2). (a) Time course (left) and quantitative values (right) of Vm in leaves treated with 3 ml of 1 µM Tet1, Tet2 or GFP protein dissolved in 5 mM MES-NaOH (pH 6.0). Data represent the mean and standard error (n = 4). (b) False-color image reconstructions of fluorochemical intracellular Ca^{2+} in leaves cut mechanically and treated by application of tetranins dissolved in HEPES

723 buffer (3 μM), with the buffer alone serving as a control. The green fluorescence refers
724 to the binding of Calcium Orange with Ca^{2+}, whereas the chloroplasts are evidenced by a
725 bright red color caused by chlorophyll fluorescence. (**c**) The tetranin-induced generation
726 of reactive oxygen species (ROS) was monitored in bean leaf discs after the application
727 of tetranin or GFP protein solution (3 μM). The upper and lower panels, respectively,
728 show the time-dependent transition and maximum accumulation of ROS in leaf discs after
729 challenge with each protein. Data represent the means and standard errors (n = 6). The
730 means indicated by different small letters are significantly different based on an ANOVA
731 with post hoc Tukey's HSD ($P < 0.05$).
732
733 **Fig. 5** Indirect plant defenses in leaves in response to tetranins. (a) The olfactory response
734 of *Phytoseiulus persimilis* when offered volatiles released by plants treated with MD +
735 tetranin or GFP protein solution (3 μM) for 24 h or plants infested with 100 mites for 24
736 h vs. untreated control plants (Ct). The figures in parentheses represent the numbers of
737 predators that did not choose either odor source ("no choice" subjects). A replicated G-
738 test was conducted to evaluate the significance of the attraction in each experiment (***,

739 $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$; ns, $P > 0.05$). (b) Headspace volatiles

740 released from plants treated with MD + GFP, Tet1 (T1) or Tet2 (T2) were collected after

741 24-27 h. Data represent the means and standard errors (n = 4-5). The means were not

742 significantly different (ns, $P > 0.05$), on the basis of an ANOVA. (*E*)-DMNT, (*E*)-4,8-

743 dimethyl-1,3,7-nonatriene; MeSA, methyl salicylate; (*E*,*E*)-TMTT, (*E*,*E*)-4,8,12-

744 trimethyltrideca-1,3,7,11-tetraene.

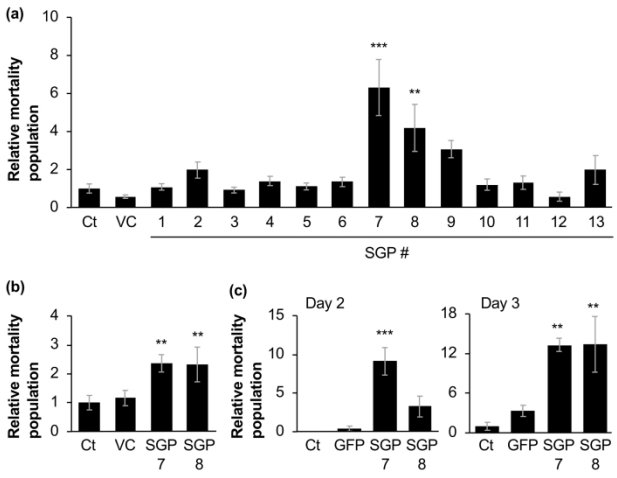

Fig. 1

Figure 1

297x209mm (300 x 300 DPI)

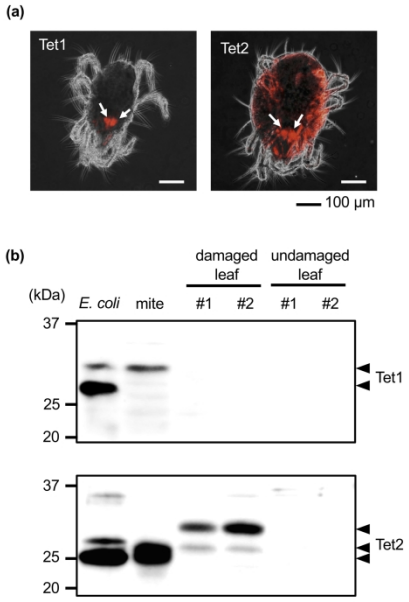

Fig. 2

Figure 2

297x209mm (300 x 300 DPI)

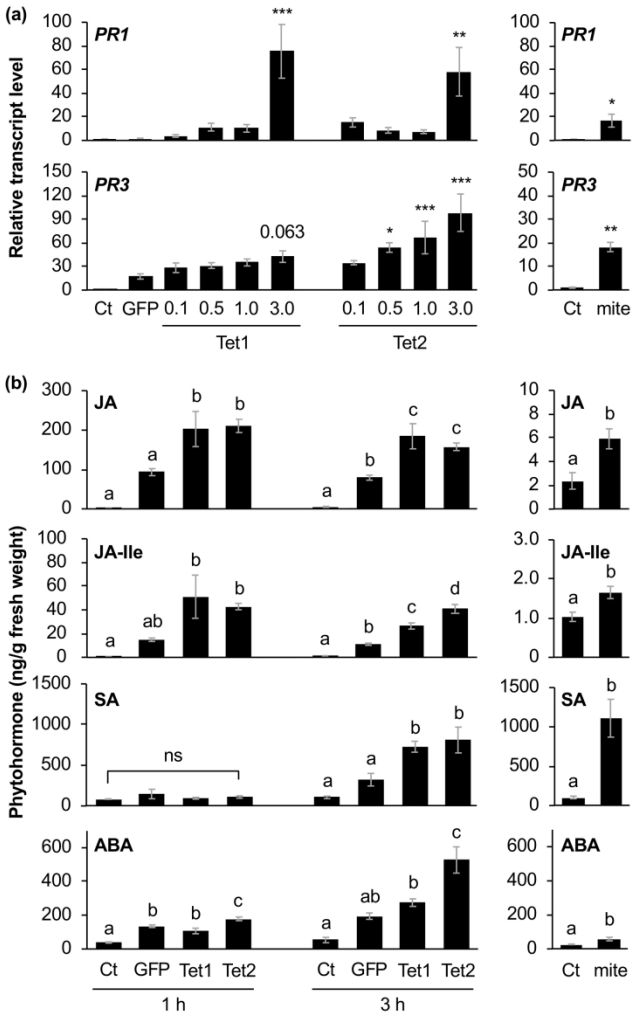

Fig. 3

Figure 3

209x297mm (300 x 300 DPI)

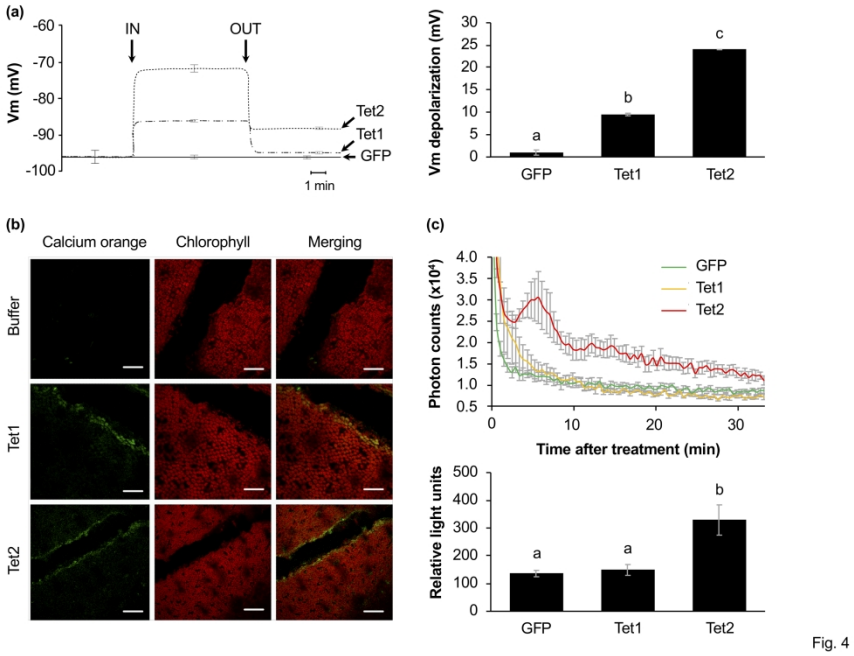

Fig. 4

Figure 4

297x209mm (300 x 300 DPI)

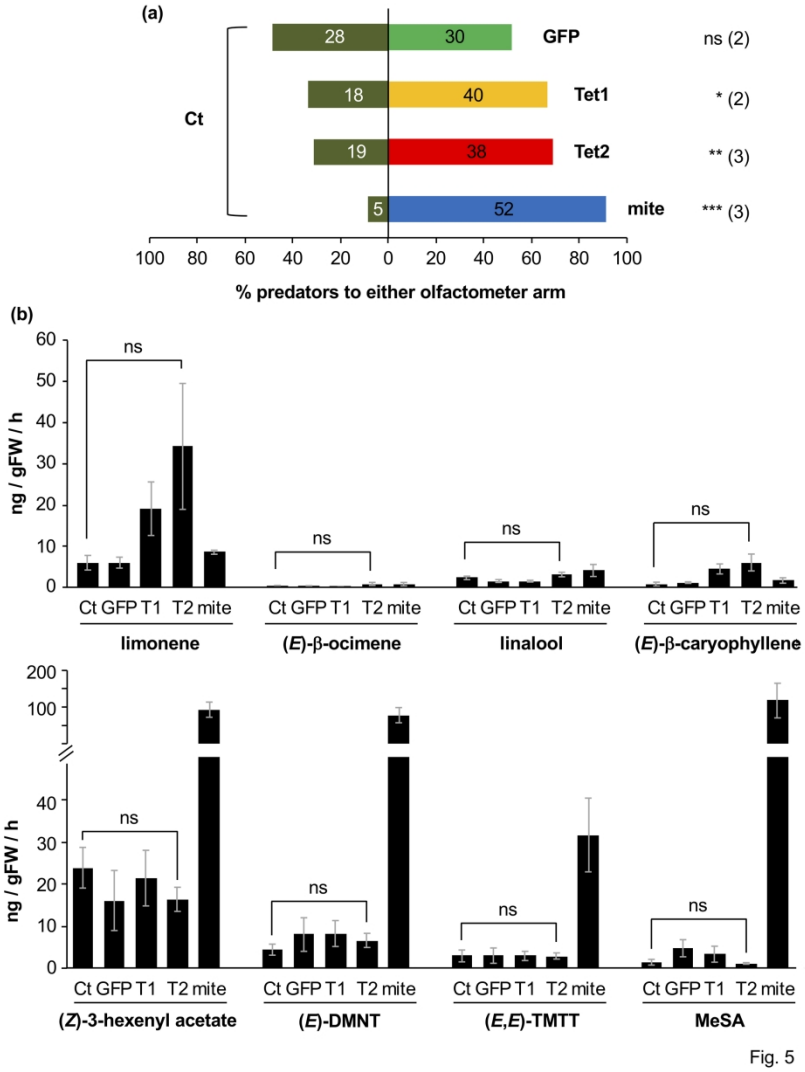

Fig. 5

Figure 5

209x297mm (300 x 300 DPI)